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Enhancing Stability and Purity of Crude Chitinase of *Achatina fulica* by Crystallization

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Abstract

A crystallization method was developed to enhance the purity and stability of hydrolase mixtures from the digestive gland of the snail *Achatina fulica*, as demonstrated by chitinase activity. Crude chitinase was concentrated by freeze drying and then crystallized at 10°C. Crystal formation was observed under the microscope. The best concentration for crystallization was obtained with 1.5-fold concentrated crude chitinase. Crystallization enhanced the chitinase specific activity from 0.87 U mg^{-1} to 0.95 U mg^{-1}. The loss of chitinase activity from liquid and crystals of crude chitinase on four days storage at 10°C was 83.0% and 17.7%, respectively. It was concluded that the crude chitinase crystals showed a significant increase in stability and purity.

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: Chitinase; crystallization; digestive gland; *Achatina fulica*; purification; stability

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1. Introduction

Snail meat is a food with a high nutritional value and has been consumed by humans worldwide since prehistoric times[1]. Many people in developing and under-developed countries use snail meat in their diet as a source of protein to raise the defense mechanisms of the body[2]. It means that snail meat is a source of nutrition, including enzymes produced in the digestive gland. The digestive gland of *Achatina fulica* produces a mixture of hydrolases, which effectively degrade the extracellular matrix polymer of fungal biofilms, especially of *Candida*. This enzyme mixture is being developed as an antifungal drug to eradicate *Candida* biofilm in all types of candidiasis pathologies, where nearly all current antifungals cannot penetrate and do not work well.

Candida species are major human fungal pathogens that contribute to human morbidity and mortality through both mucosal and deep tissue infections. As with other microbiomes[4,5], the majority of *Candida* biofilms contributes to human diseases, like autism[3] and degenerative diseases. Biofilms are biological communities with a high degree of organization, in which microorganisms form structured, coordinated and functional biological communities. These communities are embedded in a self-created extracellular matrix polymer[6]. The fungal cell walls are predominantly composed of β-1,3-glucan, β-1,6-glucan, chitin, and mannoprotein. These components increase considerably in the biofilm matrix, and become a barrier to any drug to penetrate the cell membrane and cytoplasm[7]. However, a variety of polysaccharide degrading enzymes occurs in snails. In particular, *Achatina fulica* has a mixture of enzymes, which potentially degrade the extracellular matrix polymer of fungal biofilms, especially *Candida*[8].

These mixtures of enzymes are unstable during room temperature storage. It is known that many enzymes show a higher stability in the crystalline form. Crystallization of proteins and enzymes is usually used to obtain a pure solid for X-ray diffraction analysis for structure determination. However, the novelty and the long-term goal of the current research is the large-scale partial purification of snail crude enzymes by crystallization for treatment of candidiasis. Crystallization is widely used in the final steps of chemical industry processing, to provide higher-purity solids that are easier to handle and store compared to liquids. Like the crystallization process of small inorganic molecules, protein crystallization goes through the three distinct stages of nucleation, crystal growth, and termination. Unlike inorganic small molecules, however, protein crystallization is very sensitive to environmental conditions due to the conformational flexibility of proteins. Protein crystallization is influenced by biological parameters such as the presence of contaminating biomolecules. Protein crystallization is also influenced by protein concentration, pH, temperature, ionic strength, purity, and viscosity. From the large-scale processing point of view, operational parameters such as protein concentration, salt type and concentration, pH, and temperature are considered critical. Salt concentration, pH, and temperature can directly alter a protein's solubility characteristics. This article reports a crystallization process at optimum protein concentration at 5-10°C.

2. Methods

2.1. Materials

The digestive enzymes of *Achatina fulica*, here in after referred to as crude chitinase, were harvested from the *digestive* gland of *Achatina fulica*, which had been incubated in a room with controlled humidity for a week. The snail shell was cleaned and broken down to shed the digestive gland liquid, and then centrifuged at 4000 rpm. The supernatant was the crude chitinase.

2.2. Preparation of supersaturated solution of crude chitinase

The crude chitinase was concentrated by *freeze-drying.* The harvested crude enzyme was directly frozen by dipping it in liquid nitrogen. It was then concentrated with a freeze dryer until we have 1.5x, 2x and 3x crude enzyme solutions. That means that we concentrated the crude enzyme to 65%, 50% and 33% of the original volume, here in after respectively called the 1.5x, 2x and 3x crude enzyme solutions.

2.3. Crystallization procedure

Each of the concentrated solutions of crude chitinase was incubated at 5-10°C until crystals grew. The formation of crystals was analysed microscopically and the chitinase activity was assayed.

2.4. Determination of chitinase unit and spesific activity

Chitinase activity was determined based on measuring the residual chitin after hydrolysis by chitinase in the crude enzyme. It was pipetted into tube reaction 200 µl crude chitinase and 800 µl chitin (1% colloidal chitin b/v in 50 mM phosphate buffer pH 7). The mixture was incubated at 37°C for 20 minutes. The residual chitin was measured turbidimetrically and the amount of hydrolysed colloidal chitin was calculated based on the following formula:

$$Hydrolyzed\ colloidal\ chitin = Initial\ colloidal\ chitin - Residual\ colloidal\ chitin$$

The chitinase unit activity was obtained by using the following formula:

$$Unit\ Activity\ of\ chitinase\ (U/ml) = \frac{Hydrolyzed\ colloidal\ chitin}{Incubation\ time} x \frac{Total\ volume}{Enzyme\ volume}$$

The protein concentration was determined by the Bradford assay method[5]. Chitinase specific activity was calculated according to the following formula:

$$Specific\ activity\ of\ chitinase\ (U/mg) = \frac{Chitinase\ activity\ (U/ml)}{Protein\ concentration\ (mg/ml)}$$

2.5. Determination of crude chitinase stability on storage

The chitinase activity of liquid, amorphous and crystallized crude chitinase was determined at several time points during cold room storage (5-10°C) for four days.

3. Results and discussion

As shown in Fig. 1, crude chitinase obtained from digestive glands of *Achatina fulica* is unstable during storage at room temperature and in the cold room at 5-10°C.

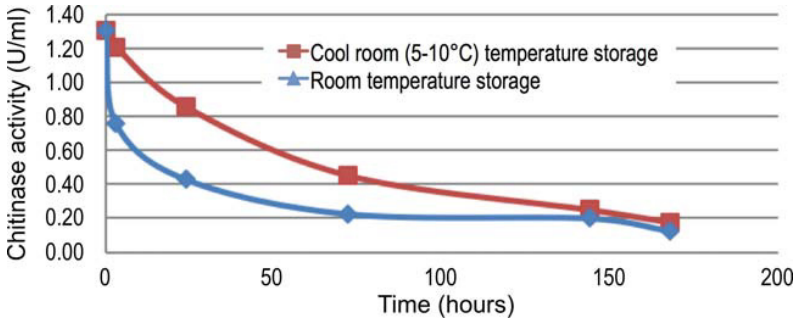

Fig. 1. Stability of crude chitinase during room temperature and cold room (5-10°C) storages.

Crystallization using enzyme solutions concentrated at 1x, 2x and 3x produced amorphous precipitate appeared as lumps under microscopic observation. Crystallization of the 1.5x concentrated enzyme solution produced crystals as presented in Fig. 2 and Fig. 3. Chitinase activity and purity of the two forms of crude chitinase compared to the initial liquid chitinase are shown in Table 1.

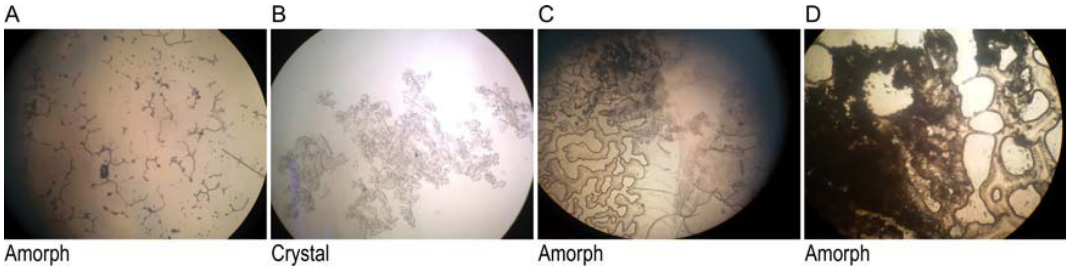

Fig. 2. Microscopic appearance of amorphous precipitates and crystals formed on crystallization of enzyme solution concentrated at (A) 1x; (B) 1.5x; (C) 2x; (D) 3x; the magnification is 100 times.

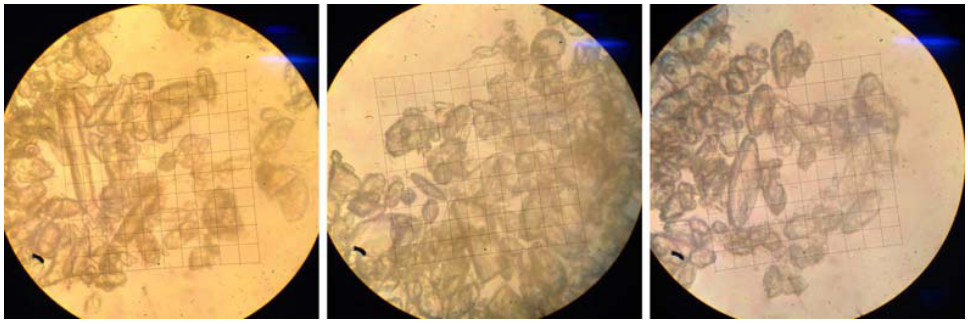

Fig. 3. Microscopic appearance of enzyme crystals produced from enzyme solution concentrated at 1.5x, observed in several sample views under 400 times magnification.

Table 1. Activity and purification level of amorphous precipitate and crystals of crude chitinase.

No.	Forms of crude chitinase	Chitinase activity (U ml^{-1})	Protein concentration (mg ml^{-1})	Specific activity (U mg^{-1})	Level of purification
1.	Liquid	1.31	1.15	0.88	1
2.	Amorphous precipitate	0.68	0.48	1.43	1.63
3.	Crystal	0.81	0.86	0.95	1.08

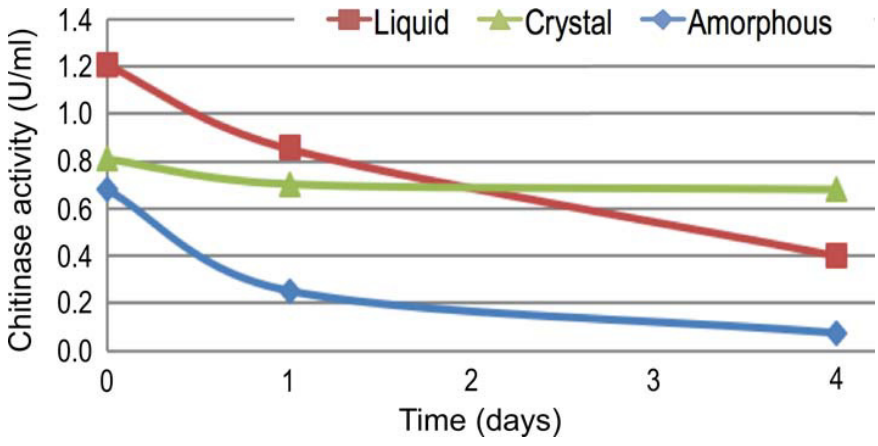

Fig. 4. Stability of liquid, amorphous and crystalline crude chitinase during cold room (5-10°C) storage for four days.

According to the data in Fig. 1, crude chitinase obtained from the digestive gland of *Achatina fulica* is a very unstable enzyme. It is shown that storage at room temperature and in the cold room resulted in a sharp drop in activity at day 2. This crucial issue can be overcome by a simple crystallization procedure for industrial application.

The initial protein concentration crucially determines the formation, purity and habits of the crystals. A low concentration of protein solution can never be crystallized or shows only a low level of crystallization with no salt added. Amorphous precipitation will occur upon salt addition, when the protein concentration is too high. Therefore, a certain, optimum protein concentration exists. It is a function of the operating variables like pH, presence of salts and temperature. The optimum protein concentration should be identified to obtain a successful protein crystallization process[9]. The crystallization process was evaluated focusing on the recovery yield (by the enzyme activity maintenance) and the crystal purity (by enzyme specific activity).

The crystallization protocol successfully removes impure proteins from the crude extract, as shown by the enhanced specific activity after crystallization. The crystallization experiments produced both amorphous precipitate and beautiful crystals of the chitinase enzyme. The enzyme purification level from the two processes was then compared. According to the data presented in Table 1, the amorphous precipitate showed a higher purification level than that of the crystallized enzyme, which means that the purity of amorphous precipitate is higher than that of the crystallized material. However, the stability test showed that the crystallized chitinase had higher stability than the amorphous one. Crystallization of the crude chitinase by evaporating the supersaturated solution enhanced the chitinase specific activity from 0.87 U mg^{-1} to 0.95 U mg^{-1}. The loss of chitinase activity from crude enzyme crystals was 17.7% in four-day storage at 10°C. The loss of chitinase activity of crude enzyme solution in one- and four-day storages at 10°C were 29.1% and 66.7%, respectively. It was concluded that the crude chitinase crystals showed significantly increased stability and purity.

Although beautiful crystals have been obtained in this work, the crystal formation process took a considerable length of time. This led to the decrease in enzyme activity. To shorten the processing time of crystallization, it is necessary to add and optimise a precipitant in the crystallization mixture in the next work.

4. Conclusions

Crystallization of the crude chitinase by evaporating the supersaturated solution enhanced the chitinase specific activity from 0.87 U mg^{-1} to 0.95 U mg^{-1}. The loss of chitinase activity from liquid and crystals of crude chitinase in four-day storage at 10°C were 17.7% and 83.0%, respectively. It was concluded that the crude chitinase crystals showed significant increase in stability and purity.

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